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Effect of *Streptococcus pneumoniae* on Human Respiratory Epithelium In Vitro

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A total of 11 of 15 *Streptococcus pneumoniae* culture filtrates and all five bacterial autolysates produced by cell death in the stationary phase caused slowed ciliary beating and disruption of the surface integrity of human respiratory epithelium in organ culture. This effect was inhibited by cholesterol and was heat labile and reduced by standing at room temperature but was stable at −40°C. The activity was detected at the late stationary phase of culture and was associated with the presence of hemolytic activity. Gel filtration of a concentrated culture filtrate and autolysate both yielded a single fraction of approximately 50 kilodaltons which slowed ciliary beating and were the only fractions with hemolytic activity. Rabbit antiserum to pneumolysin, a sulfhydryl-activated hemolytic cytotoxin released by *S. pneumoniae* during autolysis, neutralized the effect of the culture filtrate on respiratory epithelium. Both native and recombinant pneumolysin caused ciliary slowing and epithelial disruption. Electron microscopy showed a toxic effect of pneumolysin on epithelial cells: cytoplasmic blebs, mitochondrial swelling, cellular extrusion, and cell death, but no change in ciliary ultrastructure. Recombinant pneumolysin (10 μg/ml) caused ciliary slowing in the absence of changes in cell ultrastructure. Release of pneumolysin in the respiratory tract during infection may perturb host defenses, allowing bacterial proliferation and spread.

Streptococcus pneumoniae is an important human pathogen. It is a constituent of the normal nasopharyngeal flora but under permissive conditions is responsible for lower respiratory tract infections, septicemia, and meningitis. The conditions required for its proliferation in and spread from the nasopharynx are unclear, but the determinants are probably several, including both host and bacterial factors. Changes in status of the specific and nonspecific defenses of the host may be important (3, 4). For example, on the one hand, pneumococcal infections are often preceded by damage to host respiratory tract epithelium by either viral infection (4, 25) or other agents, and acquisition of a pneumococcal serotype not previously encountered may permit infection owing to absence of specific antibody (4). On the other hand, the bacterium itself elaborates a number of proteins which have been implicated in its virulence (22). Meningitis and otitis media usually occur as sequelae to respiratory tract infections which are presumably accompanied by a transient bacteremia. Bacterial factors which permit invasion of the epithelium and thus entry into the bloodstream are poorly understood.

In this study, we examined the effect of bacterial culture filtrates on human respiratory epithelium in vitro and characterized one moiety responsible for the changes observed. Pneumolysin, a thiol-activated membrane-damaging toxin, caused ciliary slowing, changes in epithelial cell ultrastructure, and disruption of epithelial integrity in a dose-dependent manner.

MATERIALS AND METHODS

Preparation of bacterial culture filtrates and autolysates. Thirteen clinical isolates of *S. pneumoniae* chosen from consecutive routine sputum cultures and two serotype 3 reference laboratory strains were incubated for 16 h at 37°C in 100 ml of broth consisting of nutrient broth number 2 (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom), 25 g/liter; liver digest, 3 g/liter; yeast extract, 3 g/liter; magnesium chloride, 0.1 g/liter; and glucose (0.15%, wt/vol). A viable count (CFU per milliliter) was performed by a standard dilutional technique and overnight incubation on blood agar. The cultures were centrifuged (5,000 × *g*) for 30 min at 4°C. The supernatants were aspirated and then filtered (0.2-μm-pore-size Acrodisc; Gelman Sciences, Inc., Ann Arbor, Mich.) to yield bacteria-free preparations. The sterility of these preparations was checked by incubation of a sample on blood agar. The pH of the culture filtrate was adjusted to 7.0 by the dropwise addition of 0.1 M sodium hydroxide to ensure that any change observed in the respiratory epithelium did not relate to the acid pH of the preparation.

An additional five broth cultures of the clinical isolates were centrifuged, and the bacterial pellet was washed in medium 199 cell culture fluid (no phenol red; Flow Laboratories, Inc., McLean, Va.), resuspended in medium 199, and left to stand at room temperature for 48 h. During this time, considerable autolysis of bacteria occurred (68% reduction in optical density), the remaining bacteria being removed by centrifugation and filtration (0.2-μm pore size). Bacterial viable counts confirmed that medium 199 did not support the growth of *S. pneumoniae*.

Effect of bacterial preparations on CBF. Strips of normal human nasal and tracheobronchial ciliated epithelium were obtained from the inferior turbinate of the nostril or the tracheobronchial mucosa during bronchoscopy by using a cytology brush (20, 25, 26). Normal volunteers for nasal brushing gave informed consent, and patients undergoing bronchoscopy for other reasons gave informed, written consent for samples to be taken from normal areas of the

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bronchial tree. These procedures were approved by the Brompton Hospital Ethics Committee. The strips of epithelium were dispersed by agitation of the cytology brush in 4 ml of medium 199, which was divided into two aliquots and centrifuged at 150 × *g* for 10 min. The supernatant medium 199 was carefully aspirated, discarded, and replaced with 200 μl of medium 199 and 600 μl of pneumococcal culture filtrate in one aliquot (test) and broth and medium 199 in the other (control). A sealed microscope cover slip-slide preparation of the resuspended epithelium was made for measurement of ciliary beat frequency (CBF) by a photometric technique (10, 25, 26). Bacterial autolysates were added to respiratory epithelium without dilution and compared with a control of medium 199 alone.

The microscope cover slip-slide preparations were randomized so that the observer was unaware of their origin and then placed on an electronically controlled warm stage (Microtec; Oxford, United Kingdom) at 37°C and mounted on a Leitz Dialux 20 phase-contrast microscope. A Leitz MPV compact microscope photometer transduced light intensity into an electrical signal. Strips of epithelium with beating cilia were viewed directly at a magnification of ×320 by bright-field illumination. The cilia were positioned to interrupt the passage of light through a small diaphragm into the photometer, and the electrical signal generated was converted into a digital reading of CBF in hertz (Hz) (10). Direct viewing of the cilia allowed an assessment of their beating pattern to be made. Ciliary dyskinesia was defined as absence of the usual paddlelike coordinated ciliary movement. Ciliary stasis was defined as complete absence of ciliary movement.

CBF was measured at hourly intervals for 4 h after initial equilibration for 10 min. Six to ten strips of ciliated epithelium were identified on the slide and marked for reidentification. Hence, CBF was recorded from as close as possible to the same point on each strip at each experimental time point. The 6 to 10 strips were all used as sites of 10 CBF readings at each time point, no more than 2 readings being taken from any one strip. The mean of the 10 readings at each time point was calculated for both control and test slides. Ciliary dyskinesia and ciliary stasis were noted if present. The CBF of static cilia was not counted as zero unless all the cilia on a selected epithelial strip ceased to move. This excluded any bias toward selecting areas of prominent slowing but tended to underestimate the potency of the bacterial preparations as it ignored static cilia when some ciliary movement remained elsewhere on the strip.

By comparing control and test mean CBF, we detected the maximum ciliary slowing for each pneumococcal strain. Percent ciliary slowing was calculated at this time point by the equation [(control CBF − test CBF)/control CBF] × 100. The 10 control readings of CBF were compared with the 10 test readings by the unpaired Student *t* test.

Production of cilioinhibitory factor during *S. pneumoniae* broth culture. The clinical isolate CS1, which was the most potent of the 15 strains tested at slowing ciliary beating, was cultured in broth for 24 h. Samples were removed at 4-h intervals for calculation of bacterial viable count and assay of ciliary slowing properties.

Characterization of factor(s) responsible for ciliary slowing. (i) The CBF assay was performed after the culture filtrate was heated to 56°C for 30 min, after the culture filtrate stood at room temperature for 24 h, and after storage of the filtrate for 1 month at −40°C.

(ii) The broth culture filtrate and the bacterial autolysate in medium 199 were both concentrated by dialysis against propylene glycol (from 100 to 5 ml) and applied to a Sephacryl S-200 Superfine (Pharmacia, Uppsala, Sweden) gel filtration column (30 by 2.5 cm; void volume, 175 ml). Phosphate-buffered saline (PBS) was used as the eluent, and fractions were stored at −40°C for testing in the CBF assay. The approximate molecular weight corresponding to the fractions was calculated with standard molecular weight markers. The hemolytic activity of each fraction was determined with serial twofold dilutions incubated for 30 min at 37°C with an equal volume of 1% packed, washed human group O erythrocytes in PBS. Hemolysis was monitored by UV absorbance of the supernatant at 541 nm (18).

(iii) The CBF assay was performed after the addition of cholesterol to the culture filtrate. A saturated solution of cholesterol (25 mg/ml) in 100% ethanol was added to the broth control or culture filtrate (1:1,000). Four preparations were tested: broth alone, broth plus cholesterol, culture filtrate alone, and culture filtrate plus cholesterol.

(iv) The CBF assay was performed with a further eight culture filtrates, and their hemolytic activity was measured simultaneously. The hemolytic activity of each filtrate was determined by a semiquantitative microdilution plate assay (16, 18). Aliquots of 50 μl of each filtrate were serially diluted in PBS and incubated with a 50-μl suspension of 1.6% fresh horse erythrocytes at 37°C for 30 min in a 96-well microdilution plate. The well showing 50% hemolysis on visual inspection was read as the endpoint. The culture filtrates were ranked separately based on their ciliary slowing activity and their hemolytic activity. The rankings were compared by the Spearman rank correlation coefficient.

Effect of pneumolysin on human ciliated epithelium. Two preparations of pneumolysin were purified from extracts of organisms grown overnight at 37°C. Recombinant pneumolysin was purified from *Escherichia coli* MC1061 (7) harboring plasmid pJW252 (22, 23), and native toxin was from *S. pneumoniae* R36A (a nonencapsulated laboratory strain). Toxins were purified by hydrophobic interaction chromatography on a TSK phenyl-5PW column by high-pressure liquid chromatography (LKB Pharmacia, Milton Keynes, United Kingdom). Crude cell extracts were produced by sonication and centrifugation and were applied to the column in Tris-buffered saline (10 mM Tris hydrochloride [pH 7.5], 250 mM NaCl) and then washed with the same buffer. Fractions were assayed for hemolytic activity. Pneumolysin was retarded on the column and was eluted in later washing fractions. Concentrated hemolytic fractions were homogeneous as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and N-terminal amino acid sequencing (16). Pneumolysin was dissolved in PBS and added to a suspension of epithelial strips in PBS (final concentration, 1 to 50 μg/ml). The effect on CBF was measured as described above and compared with that of a control in PBS alone.

Serum was obtained from rabbits before and after immunization with pneumolysin. Pneumolysin (10 μg) in Freund complete adjuvant was injected intramuscularly into rabbits once weekly for 3 weeks. Two weeks later, the rabbits received an intravenous booster injection of 10 μg of pneumolysin and were bled from the ear vein 1 week later. Blood was clotted overnight at 4°C, and collected serum was stored at −20°C in aliquots until use. Three preparations were compared: first, 100 μl of immune serum added to 500 μl of *S. pneumoniae* CS1 culture filtrate; second, 100 μl of normal rabbit serum collected prior to immunization added to 500 μl of *S. pneumoniae* CS1 culture filtrate; and third, 100 μl of normal rabbit serum added to broth alone. Previous experiments had shown that serum itself did not affect CBF (after

4 h, broth CBF = 13.6 Hz, broth + serum CBF = 13.6 Hz). All three preparations were incubated at 37°C for 30 min and then added to ciliated epithelium for the CBF assay. The same experiment was repeated with the serum of a horse immunized with streptolysin O (kindly provided by J. Alouf, Pasteur Institute).

TEM. The effect of recombinant pneumolysin on the ultrastructure of ciliated epithelium was studied by transmission electron microscopy (TEM) (25). Experiments were repeated as described above, incubating epithelium with different test solutions at 37°C. After 4 h, the epithelium was transferred to cacodylate-buffered 2.5% gluteraldehyde and postfixed in 1% osmium tetroxide. After rinsing, the brushings of ciliated epithelium were embedded in a drop of 2% liquid agar and gently centrifuged. The agar was allowed to solidify and then routinely processed through to embedding in Araldite. Semithin (1 μm) sections were cut and stained in 1% toluidine blue for light microscopy, and suitable areas were selected and trimmed for ultrathin sectioning. These ultrathin sections were stained with uranyl acetate and lead citrate for TEM. Sections were assessed by an observer unaware of their origin, and the following abnormal features were recorded morphometrically: projection of cells from the surface of the epithelium, cytoplasmic blebs from the cell surface, evidence of mitochondrial damage, and the number of very unhealthy or dead cells. The number of cells with absent or reduced ciliation was compared with the number of fully ciliated cells. Ciliary abnormalities were recorded as being either microtubular or of the dynein arms. All results were expressed with reference to the total number of cilia or cells counted.

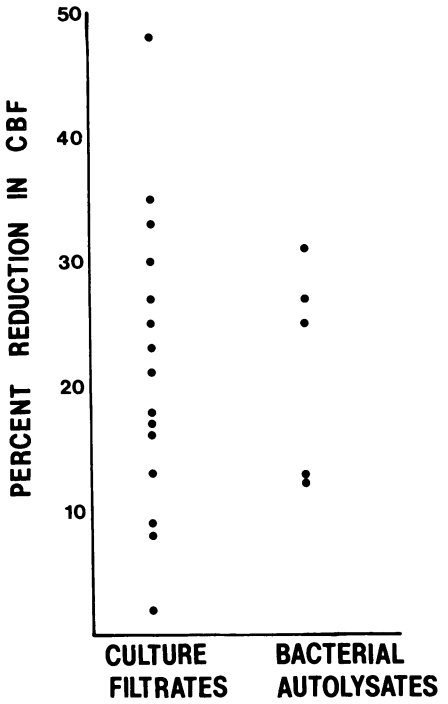

FIG. 1. Effect of bacterial culture filtrates and bacterial autolysates on CBF of human respiratory epithelium in vitro. The minimum mean CBF obtained during an experiment is expressed as the percent reduction of control CBF at the same experimental time point.

RESULTS

Of 15 *S. pneumoniae* broth culture filtrates, 11 caused significant ($P < 0.001$) slowing of CBF (Fig. 1). The effect of culture filtrates on respiratory epithelium obtained from the inferior turbinate of the nostril or from the bronchial tree was identical. Ciliary slowing was of immediate onset and progressed during the first hour of the experiment, CBF continuing to decrease or remaining constant during the remaining 3 h of the experiment. Ciliary dyskinesia was only occasionally observed and then only when there had been substantial CBF slowing, whereas areas of ciliary stasis were observed more frequently. During the 11 experiments in which significant ciliary slowing occurred, the epithelial surface became disrupted (loss of the integrity of the epithelial surface, which became broken and irregular), in contrast to control experiments, during which it remained regular.

Medium 199 did not support the growth of *S. pneumoniae*; thus, the autolysis preparations contained predominantly products of bacterial breakdown alone. Each of the five autolysates prepared, from five strains that had produced active culture filtrates, caused significant ($P < 0.001$) slowing of CBF (Fig. 1), and the changes observed in CBF and epithelial structure were of a magnitude similar to and indistinguishable from those produced by broth culture filtrates.

The filtrates of samples taken during a 24-h broth culture of *S. pneumoniae* CS1 only slowed ciliary beating after 12 h of growth (Fig. 2) and did not cause slowing when obtained during log-phase growth. The activity continued to increase until 16 h into the growth curve, at which time the bacterial viable count had begun to decrease. Subsequently, the level of ciliary slowing activity waned, suggesting that the active factor(s) causing ciliary slowing was either labile or had been neutralized or degraded within the broth. The level of ciliary slowing activity correlated closely with the hemolytic activity of the culture filtrate (Fig. 2). The delayed development of ciliary slowing activity in broth culture was consistent with the notion that death of bacteria led to release of a ciliary slowing factor.

The ciliary slowing activity of culture filtrates was abolished by heating to 56°C for 30 min and was reduced (by 35%) by standing at room temperature for 24 h. This lability could partly explain the reduction in activity after 24 h in culture (Fig. 2). However, activity was completely preserved after storage for 1 month at −40°C. Gel filtration of both the concentrated culture filtrate and autolysate of *S. pneumoniae* CS1 yielded a single fraction which produced significant ($P < 0.01$) slowing of CBF (Fig. 3). In each case, the active fraction was approximately 50 kilodaltons and possessed hemolytic activity which was absent from all other fractions (Fig. 3).

The addition of cholesterol (25 μg) to the broth did not affect CBF but markedly reduced ciliary slowing activity in the culture filtrate (by 45%). Because the cholesterol was dissolved in ethanol (which is itself toxic to cilia), larger concentrations could not be used. There was excellent correlation ($r = 0.98$) between hemolytic activity and ciliary slowing activity measured simultaneously in eight culture filtrates.

The pneumococcus produces a thiol-activated membrane-damaging toxin which is released during cell death. It is hemolytic, and its activity is neutralized by heating at 56°C and by cholesterol. To test this, we obtained purified pneumolysin from two sources: *S. pneumoniae* and *E. coli* carrying the cloned pneumolysin gene. Pneumolysin was measured by weight rather than by hemolytic titer, as the

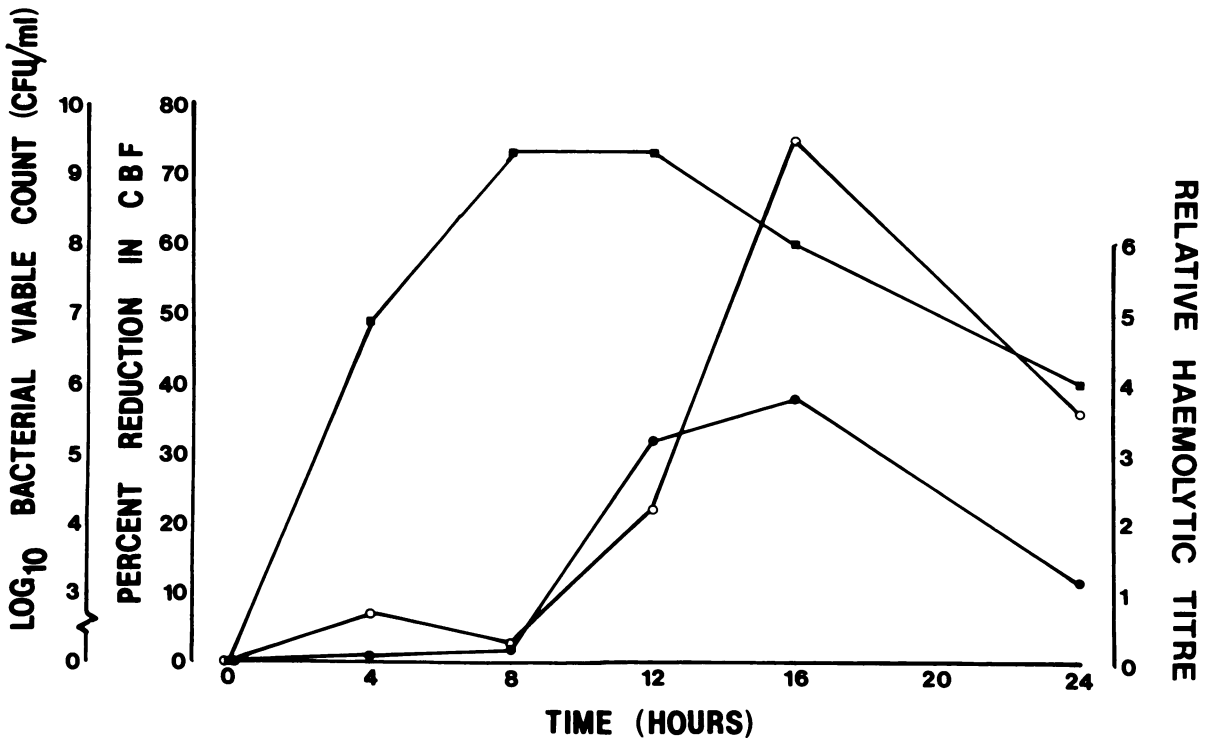

FIG. 2. Effect of samples taken during a 24-h broth culture of *S. pneumoniae* on CBF of human respiratory epithelium in vitro. The bacterial viable count (CFU per milliliter) obtained from each sample (■), the effect on CBF of each sample after filtration (○), and the hemolytic activity of the sample (●) are shown at each time interval.

latter was found to vary depending on the batch of erythrocytes and their age (data not shown). In the semiquantitative hemolytic assay, native pneumolysin was found to have a hemolytic titer of between 5×10^5 and 1×10^6 hemolytic units per mg of protein. Although this variation meant that hemolytic titer could not be used to calculate accurately the amount of pneumolysin present in biological preparations, it did not invalidate the use of a single batch of erythrocytes to compare the relative activity of different samples of pneumolysin. Native pneumolysin (10 μg/ml, final concentration) was added to broth, and this produced 50% ciliary slowing after 4 h when compared with control broth alone. The ciliary slowing and disruption of the epithelial surface produced were indistinguishable from that observed with culture filtrates. The addition of pneumolysin immune rabbit serum to broth culture filtrate completely neutralized the ciliary slowing effect: control slide, 12.7 Hz; culture filtrate with normal rabbit serum, 7.8 Hz; culture filtrate with immune rabbit serum, 12.3 Hz. Similar neutralization was obtained with an antibody raised in rabbits against streptolysin O. However, some reduction in ciliary slowing activity was noted with the addition of normal rabbit serum, so that other factors in serum (such as cholesterol) may inhibit ciliary slowing activity.

A series of dose-response experiments were performed with each preparation of pneumolysin in PBS (Fig. 4). In each case, dose-dependent ciliary slowing was produced. The pneumolysin purified by high-pressure liquid chroma-

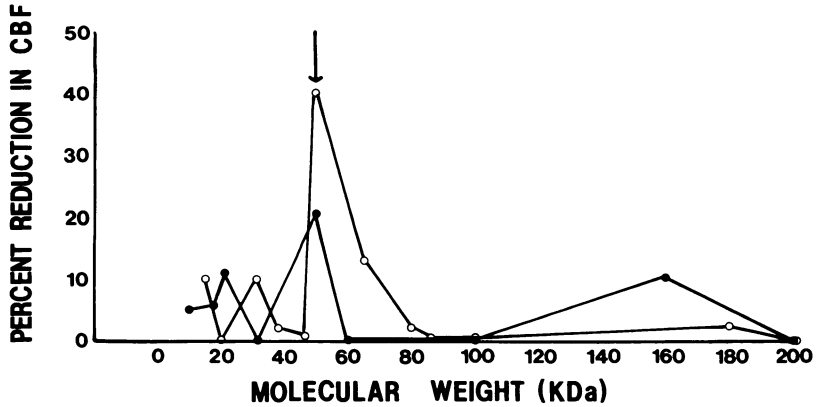

FIG. 3. Effect of fractions obtained by gel filtration of an *S. pneumoniae* culture filtrate (○) and an *S. pneumoniae* autolysate (●) on CBF of human respiratory epithelium in vitro. Hemolytic activity was only detected in a single fraction of each preparation (arrow).

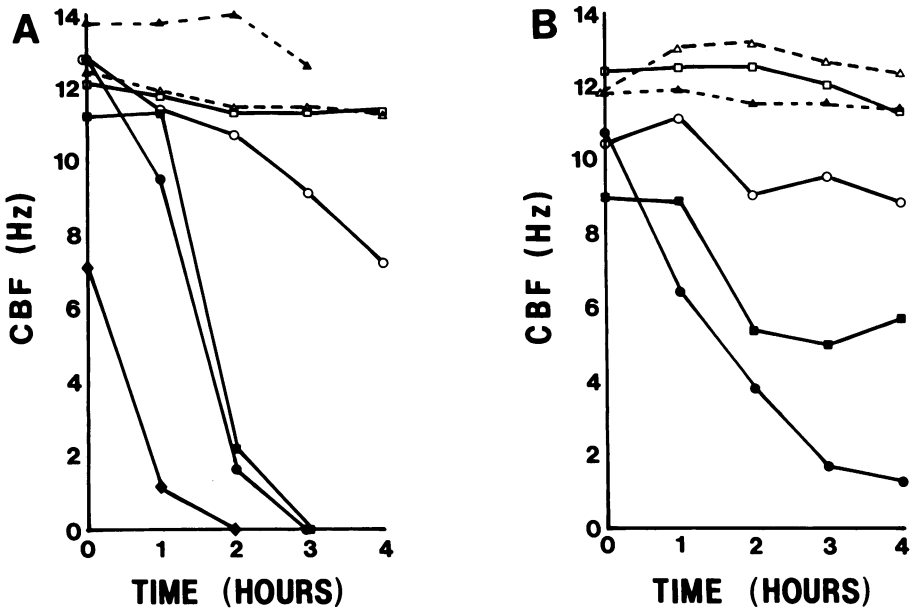

FIG. 4. (A) Effect of native pneumolysin purified by high-pressure liquid chromatography from *S. pneumoniae* on CBF of human respiratory epithelium in vitro. Open and solid symbols represent data obtained from different biopsies. Symbols: experiment 1, ▲, control (PBS); ◆, 50 μg of pneumolysin per ml; ●, 25 μg of pneumolysin per ml; and ■, 10 μg of pneumolysin per ml; experiment 2, △, control (PBS); ○, 5 μg of pneumolysin per ml; □, 1 μg of pneumolysin per ml. (B) Effect of recombinant pneumolysin (purified by high-pressure liquid chromatography from *E. coli* MC1061 harboring the plasmid pJW252 carrying the pneumolysin gene) on CBF of human respiratory epithelium in vitro. Symbols: experiment 1, ▲, control (PBS); ●, 50 μg of pneumolysin per ml; ■, 25 μg of pneumolysin per ml; experiment 2, △, control (PBS); ○, 10 μg of pneumolysin per ml; □, 5 μg of pneumolysin per ml.

tography from *S. pneumoniae* affected CBF more than the recombinant preparation, although the difference between the two preparations was within the batch-to-batch variation we have found in hemolytic activity of pneumolysin and is probably not significant. Native pneumolysin caused complete ciliary stasis and epithelial disruption (10 μg/ml), and significant ciliary slowing ($P < 0.001$) occurred at lower concentrations (5 μg/ml). The ciliary slowing activity of native pneumolysin (10 μg) was greater in PBS than in broth, perhaps because of inhibitory factors such as cholesterol in broth.

TEM studies showed that recombinant pneumolysin caused epithelial cell damage (Table 1). An increased number of single dead and unhealthy cells were observed, and indications of cell injury (cytoplasmic blebs and mitochondrial swelling) were noted more frequently within the epithelial strip. Cytoplasmic blebs were seen (in cross-section) to project between the ciliary axonemes and could therefore possibly cause physical interference with ciliary function. The changes observed (Fig. 5) were dose dependent but only present at the higher concentrations tested (25 and 50 μg/ml). The disrupted epithelial surface seen by light microscopy was shown by TEM to represent projection of epithelial cells from the epithelial surface and separation of the tight junctions between cells. The ultrastructure of cilia on living cells was unaffected, and the extent of ciliation of the epithelium was unchanged by treatment with pneumolysin. Recombinant pneumolysin (10 μg/ml), a concentration which caused ciliary slowing without epithelial disruption being observed by light microscopy (Fig. 4), did not cause any ultrastructural changes in the epithelial cells.

DISCUSSION

The production of factors which affect ciliated epithelium has been noted in several bacterial species which colonize mucosal surfaces (24). It has been suggested that such compounds play a role in the pathogenesis of respiratory tract infections. For example, by disturbing ciliary function, by stimulating mucus production (1), and by disrupting the integrity of the epithelium, they may create a favorable environment for bacterial colonization. Subsequently, they may facilitate proliferation, spread, and invasion of the subepithelial layers. To assess their in vivo relevance, the identity of these compounds must be elucidated.

TABLE 1. Effect of recombinant pneumolysin on the ultrastructure of human ciliated epithelium in vitro[a]

Feature	% of cells in control (total)	% of cells (total) at a pneumolysin concn (μg/ml) of:			
		5	10	25	50
Cells projecting from the epithelium	5.3 (774)	5.7 (315)	4.6 (603)	17 (75)	9.9 (71)
Cells with cytoplasmic blebs	10.5 (839)	17.1 (322)	11.0 (619)	22.4 (125)	18.3 (82)
Cells with mitochondrial swelling	5.8 (839)	6.5 (322)	4.2 (619)	11.2 (125)	11.0 (82)
Unhealthy or dead cells	5.1 (871)	10.8 (361)	6.1 (670)	28.8 (156)	32.8 (122)
Cells with reduced ciliation	13.6 (871)	14.6 (358)	8.8 (670)	14.5 (151)	9.1 (121)

[a] Each score represents the number of cells showing a particular feature expressed as a percentage of the total number of cells counted (shown in parentheses).

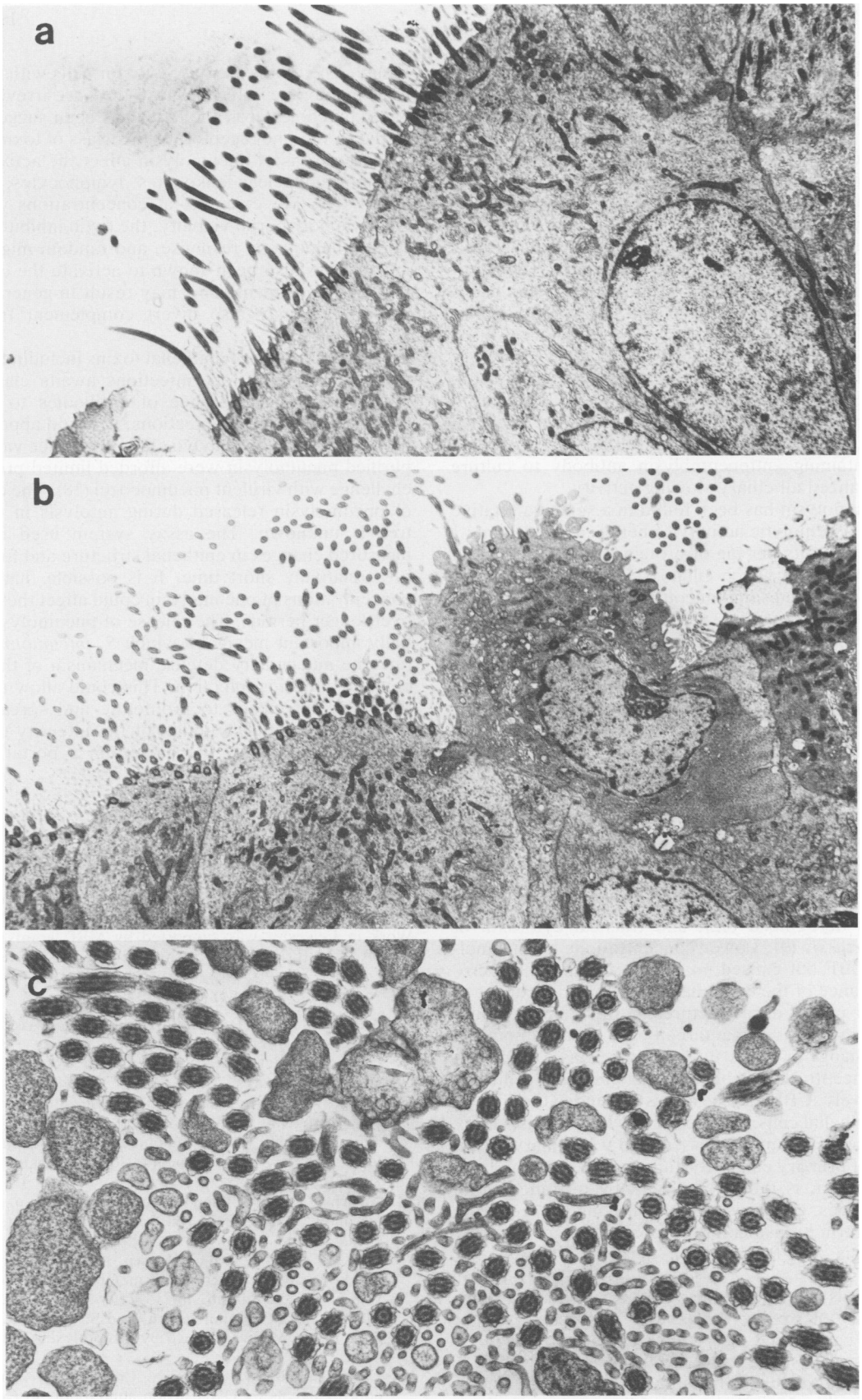

FIG. 5. Effect of recombinant pneumolysin on the ultrastructure of human respiratory epithelium examined by TEM. (a) After incubation in PBS for 4 h, the appearance of the epithelium was normal (×9,000). (b) After incubation with recombinant pneumolysin (25 μg/ml), an epithelial cell is shown protruding from the epithelial surface, cytoplasmic blebs are seen on the epithelial surface, and mitochondrial swelling is present (×9,000). (c) Cross-section through cilia from epithelium treated with recombinant pneumolysin (25 μg/ml). The ciliary ultrastructure was normal, but cytoplasmic blebs project between the ciliary axonemes (×24,500).

We showed in this study that *S. pneumoniae* culture filtrates affected human respiratory epithelium by slowing ciliary beating, causing ciliary stasis, and disrupting epithelial integrity. Ciliary slowing activity was found only in the culture filtrates of 11 of 15 strains. This might be explained by the variable production of pneumolysin by strains in culture (15), a certain level of pneumolysin being required to be present to produce an effect on epithelial cells. The ciliary slowing factor was released during the late stationary phase of broth culture and during autolysis of bacteria. The factor was heat labile, was inhibited by cholesterol, and had a molecular weight of about 50,000, and an association was demonstrated between hemolytic activity and ciliary slowing activity. These properties led us to suspect that the ciliary slowing activity was related to the action of pneumolysin on ciliated epithelium. Native pneumolysin added to fresh broth (10 μg/ml) reproduced the ciliary beat slowing and epithelial disruption caused by culture filtrates. Furthermore, addition of serum containing antipneumolysin antibody to culture filtrates neutralized all ciliary slowing activity.

Native pneumolysin has been found in a semiquantitative assay to have a hemolytic activity of between 5×10^5 and 1×10^6 hemolytic units per mg of protein (16). Extrapolating this to experiments in which ciliary slowing and hemolytic activity were measured simultaneously, the amount of pneumolysin present in a culture filtrate causing 39% ciliary slowing was about 1 μg/ml, and that in a culture filtrate causing 75% slowing was about 8 μg/ml. These estimates of the amount of pneumolysin present in culture filtrates were confirmed by an enzyme-linked immunosorbent assay.

The effect of pneumolysin on epithelial cells appeared to be mediated by its cytotoxic properties (2, 22). However, pneumolysin also caused slowing of CBF in the absence of ultrastructural changes in the epithelial cells, suggesting that more subtle changes were occurring. Pneumolysin caused extrusion of cells from the epithelial surface similar to the response seen after treatment with other bacterial toxins: endotoxin of *Haemophilus influenzae* (13) and *Neisseria gonorrhoeae* (11), rhamnolipid (heat-stable hemolysin) of *Pseudomonas aeruginosa* (12), and tracheal cytotoxin of *Bordetella pertussis* (9). Lower concentrations of pneumolysin slowed CBF but caused no change in the light microscopic appearance of the epithelial surface and no change in the cellular or ciliary ultrastructure. At this concentration, pneumolysin may be acting directly on ciliary function, although no change in ciliary ultrastructure was observed at the higher concentrations which caused epithelial cell damage. Alternatively, CBF may be a sensitive indicator of toxic changes in epithelial cells. In favor of this hypothesis, ciliary beat slowing was infrequently associated with ciliary dyskinesia, a sign of primary ciliary dysfunction (24). Streptolysin O, a related toxin, is thought to work by oligomerization within membranes, giving rise to arc- and ring-shaped structures which form transmembrane pores and in this way cause cell lysis (6). Pneumolysin may act by a similar mechanism.

Pneumolysin is a sulfhydryl-activated toxin that is a member of a family of toxins produced by diverse genera of gram-positive bacteria (2, 22). Unlike the other sulfhydryl-activated toxins, it is not secreted by the bacterium but is released during autolysis. This family of toxins shares common physical and biological properties and induces antibodies that are broadly cross-reactive and which will neutralize other toxins of the family. It was not surprising, therefore, that ciliary slowing activity was neutralized by antibody raised against streptolysin O, another member of the same family. The toxins are only lytic for cells with cholesterol in their membranes, and because they are irreversibly inactivated by cholesterol, the latter has been suggested to serve as the membrane receptor for this class of toxin (2). Sublytic concentrations of pneumolysin affect the activity of human polymorphonuclear leukocytes, lymphocytes, and platelets (8, 14, 17). For example, at concentrations which had no effect on polymorph viability, the toxin inhibited respiratory burst, chemotactic response, and random migration. Pneumolysin has also been shown to activate the classical pathway of complement. This may result in generalized inflammation and may also divert complement from invading bacteria (5, 19).

The precise role of epithelial toxins including pneumolysin in bacterial respiratory infections awaits clarification. As judged by the appearance of antibodies to pneumolysin during pneumococcal infections, it would appear that pneumolysin is produced in vivo (21). Also, mice vaccinated with purified pneumolysin were afforded limited protection from challenge with virulent pneumococci (18). The concentration of pneumolysin released during autolysis in the bronchial tree is unknown. The assay system used in this study measured changes in epithelial structure and function over 4 h, a relatively short time. It is possible that much lower concentrations of pneumolysin could affect these parameters over longer periods. The release of pneumolysin is a potentially important means by which *S. pneumoniae* could perturb the mucociliary defense mechanism of the respiratory epithelium during infection. This could allow the bacterium the time and means to proliferate and spread within the respiratory tract. Disruption of the integrity of the epithelium may also allow the bacterium a portal of entry for invasion.

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